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LOCALIZATION OF *LIMULUS POLYPHEMUS* OXYGEN SENSITIVITY

CHARLES H. PAGE

Department of Zoology, Ohio University, Athens, Ohio 45701 and the Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Rhythmic metachronal movements of the five book gill-bearing opisthosomal appendages provide respiratory ventilation in the horseshoe crab, *Limulus polyphemus* (Hyde, 1893; Waterman and Travis, 1953). Two book gills are situated on the lateral posterior surfaces of each pair of fused gill appendages. The first pair of opisthosomal appendages, the genital operculum, moves in concert with the first through fifth pairs of gill appendages. Ventilation results from the alternate abduction and adduction of each paired gill appendage. Each wave of abduction begins in the fifth gill appendage and sweeps anteriorly; abduction is closely followed by an anterior sweeping wave of adduction.

The rate and amplitude of the opisthosomal ventilatory movements are dependent upon the oxygen concentration in the sea water environment (Hyde, 1906; Waterman and Travis, 1953). Under anoxic conditions ventilation ceases. When oxygen is introduced into anaerobic sea water, there is an immediate resumption of ventilation suggesting that external oxygen receptors are present (Waterman and Travis, 1953). Previous attempts to identify these external receptors have been unsuccessful (Waterman and Travis, 1953; Schlein and Barber, 1971).

This report describes experiments in which the external sites of oxygen sensitivity were identified by examining the effects which sectioning nerves and otherwise inactivating possible sensory structures had upon the oxygen dependent ventilatory reflexes. The results demonstrate that oxygen responsiveness of the *Limulus* ventilatory system is dependent upon the integrity of the opisthosomal book gills (Fig. 1B) and the prosomal intercoxal sensory cuticle (Fig. 1C). Generation of the ventilatory rhythm in intact animals is dependent upon afferent input from these structures.

All appendages and sensory structures to which reference is made in the text of this report are labeled in Figure 1.

Methods

Adult and immature (prosoma widths of 6–8 and 3–4 inches respectively) specimens of *Limulus polyphemus* (L.) were obtained from the Supply Department, Marine Biological Laboratory, Woods Hole, Massachusetts. Upon receipt the telsons were removed. Animals were fed pieces of beef liver and maintained for 1–3 months in a circulating sea water tank (at the MBL) or in a 120 gallon aquarium containing Dayno synthetic sea water (at Ohio).

Animals were placed in a sea water-filled plexiglass chamber and secured in an extended position, ventral side up, with four hooks inserted through the margins of the prosomal and opisthosomal carapace (Fig. 1A). Air, nitrogen and oxygen

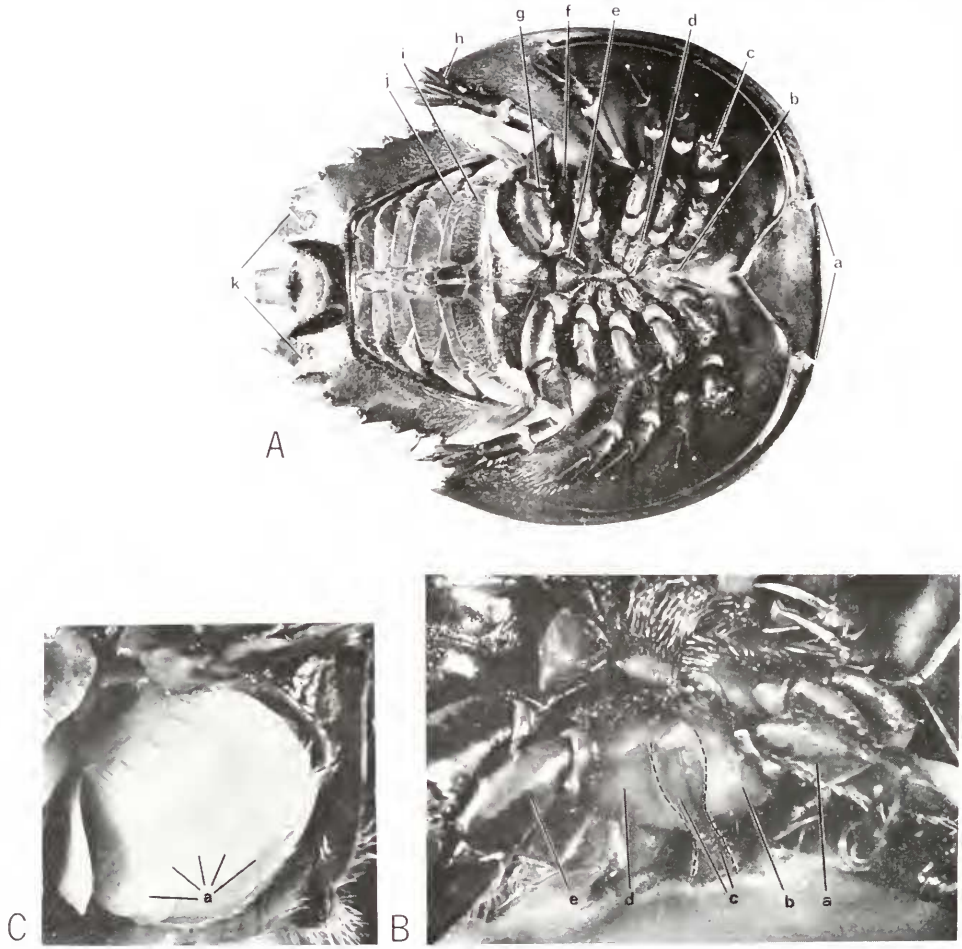

FIGURE 1. Appendages and regions of oxygen sensitivity in an adult *Limulus*: (*A*) Ventral view. Symbols used are: a = hooks securing prosoma; b = ventral eye; c = 1st walking leg (w.l.); d = gnathobase spines of 2nd w. l.; e = chilaria; f = flabellum of 5th w. l.; g = basipodite of 5th w. l.; h = spatulate podite appendages of 5th w. l.; i = operculum; j = 1st gill appendage; k = hooks securing opisthosoma. (B) is a view of intercoxal sensory cuticle. Symbols used are a = basipodite of 3rd w. l.; b = coxa of 3rd w. l.; c = intercoxal cuticular membranes (enclosed by dashed line); d = coxa of 4th w. l.; e = basipodite of 4th w. l. (C) is a view of book gill on posterior surface of 1st gill appendage; a = lamellae of book gill.

gases were introduced into the sea water through air stones placed in the chamber corner adjacent to the anterior left edge of the carapace. The experimental procedure was to seal the chamber with a sheet of Parafilm, displace the oxygen from the air-saturated sea water by bubbling nitrogen into the chamber until the sea water was sufficiently anaerobic (usually less than 2×10^{-4}% O_2), turn the nitrogen off and on several times to control for the possibility of the animal responding to

changes in the flow of gas bubbles from the air stones and then introducing oxygen while recording the response of the animal.

Oxygen levels of the sea water were monitored with an oxygen electrode (Yellow Springs Instrument Co. #5418) located in the posterior right chamber corner. All oxygen measurements were in parts per thousand (‰) corrected for temperature and salinity with the #51A oxygen meter. The temperature range was 19°–22° C.

Movements of the gill appendages were monitored by differential recording between two insulated 100 μm copper wires—one affixed to the side of the experimental chamber and the other attached to the exopodite of the first gill appendage. For recording muscle activity, bipolar electrodes—made by cementing together two 100 μm insulated copper wires—were inserted into the extensor muscle of the basipodite of the fifth walking leg and into either the abductor or adductor muscles of the first gill appendage. With these electrodes movement artifacts were minimized.

All potentials were differentially amplified with a Grass P15 preamplifier, fed into a Physiograph DC amplifier and recorded with a Physiograph pen recorder.

Prosomal and opisthosomal components of the ventilatory reflex were differentiated in immature animals whose opercular nerves and ventral nerve cords were sectioned immediately posterior to the chilaria. The opercular nerves and ventral nerve cord exit from the nerve ring and run posteriorly to the operculum and more posterior opisthosomal segments respectively (Patten and Redenbaugh, 1899). The surgical procedure was to make an incision in the cuticle between the chilaria and operculum exposing the opercular nerves and ventral nerve cord and then to section them. In addition, in several animals the first dorsal nerves, which arise from the anterior ventro-lateral aspect of the nerve ring and run posterior to innervate the ventral cuticle in the opisthosoma (Patten and Redenbaugh, 1899) were exposed through an incision in the dorsal prosomal cuticle slightly medial to the lateral eye and sectioned.

Animals whose opercular nerves and ventral nerve cords had been sectioned were allowed a 3 week recovery period before their ventilatory reflexes were examined. Although prosomal-opisthosomal coordination was absent, the activity levels and responsiveness of these animals appeared normal. After completion of the experiment surgical interruption of the sectioned nerves was checked by dissection.

A series of experimental procedures were employed to localize those sensory structures which are concerned with oxygen ventilatory reflex responsiveness. These structures were either surgically removed, destroyed by cauterization or inactivated by covering them with a layer of low melting point wax (Tackiwax, Cenco). The question of the involvement of book gill receptors in the oxygen ventilatory reflex was examined by monitoring the effects of each of the following experimental procedures upon the oxygen ventilatory reflex: (i) covering the book gill lamellae with wax; (ii) sectioning the gill nerve just proximal to the point at which it innervates the book gill lamellae (Patten and Redenbaugh, 1899); (iii) careful surgical removal of all book gill lamellae which can be accomplished with only slight blood loss—perhaps the dorsal abdominal flexors close off the afferent branchial arteries under these conditions (Lochhead, 1950).

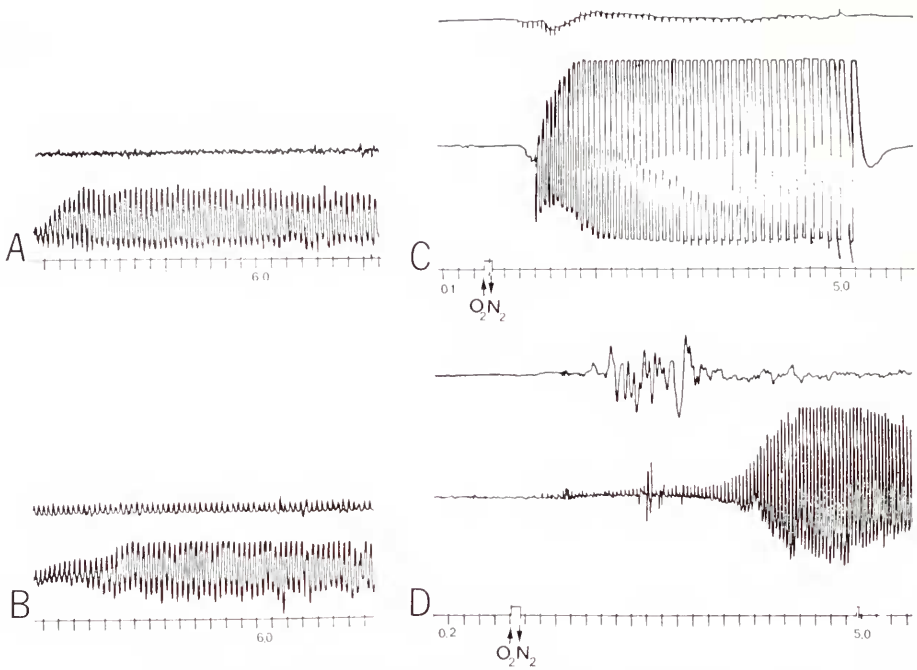

FIGURE 2. *Limulus* ventilatory reflexes. (A) Respiratory movements of gill appendages. Note absence of walking leg movement. (B) Coupled swimming movements of walking legs and gill appendages. (C) Walking leg and gill appendage responses to the introduction of oxygen into the anaerobic chamber. (D) Oxygen responsiveness of walking legs and gill appendages in an animal whose opercular nerves and ventral nerve cord were previously sectioned between the prosoma and opisthosoma; upper trace: recording of movement (A and B) or basipodite muscle responses (C and D) of 5th walking leg; middle trace: recording of movement (A and B) or adductor muscle responses (C and D) of 1st gill appendage; lower trace: 5 sec time marks; all measurements of oxygen are in $\%_{o} \times 10^{-3}$; animals were immature. These records have been retouched for photographic reproduction.

RESULTS

Low amplitude metachronal respiratory movements of the opisthosomal appendages (operculum and 5 pairs of gill appendages) usually served to ventilate the book gills of adults secured in the experimental chamber (Fig. 2A). However on occasion, intermittent bouts of swimming—metachronal movements of the opisthosomal appendages coupled with rhythmic movements of the prosomal walking legs—were observed (Fig. 2B). Although swimming in adults was intermittent, periods of sustained swimming were often observed in immature animals.

The respiratory rate was proportional to the logarithm of the oxygen concentration from 1×10^{-4} to 2×10^{-3} $\%_{o}$ O_2 (Fig. 3). Observed respiratory rates ranged from 5 per min (below 3×10^{-4} $\%_{o}$ O_2) to 40 per min (above 2×10^{-3} $\%_{o}$ O_2). Respiratory movement amplitude also decreased as the oxygen level was lowered to 5×10^{-4} $\%_{o}$ O_2.

With sufficient exposure to anaerobic conditions ventilatory movements (res-

piration and swimming) always ceased. In agreement with Waterman and Travis (1953) when oxygen was introduced into the anaerobic sea water, ventilation would resume in 5 to 60 seconds. Rhythmic metachronal movements of the opisthosomal appendages, evoked by the introduction of oxygen, were usually accompanied by movements of the prosomal walking legs (Fig. 2C). Occasionally these leg movements were nonrhythmic; at other times they were rhythmic and coupled with opisthosomal appendage beating, resembling normal swimming movements. Resumption of ventilation was in response to the appearance of oxygen; not to physical vibration of the water resulting from turning the oxygen inflow on. Turning nitrogen inflow off and on again usually elicited no walking leg or gill appendage responses (Fig. 4C) although on occasion a short duration bout of low frequency movements was observed (see also Waterman and Travis, 1953).

Prosomal walking leg and opisthosomal appendage responsiveness to the introduction of oxygen into the anaerobic chamber could be differentiated in animals whose opercular nerves and ventral nerve cords had been sectioned between the prosomal chilaria and the opisthosomal operculum. Five to 60 seconds following the introduction of oxygen into the anaerobic chamber rhythmic movements of the gill appendages and nonrhythmic movements of the prosomal walking legs were invariably initiated (Fig. 2D).

Oxygen responsiveness of the gill appendages in animals with sectioned opercular nerves and ventral nerve cords depends upon the integrity of the book

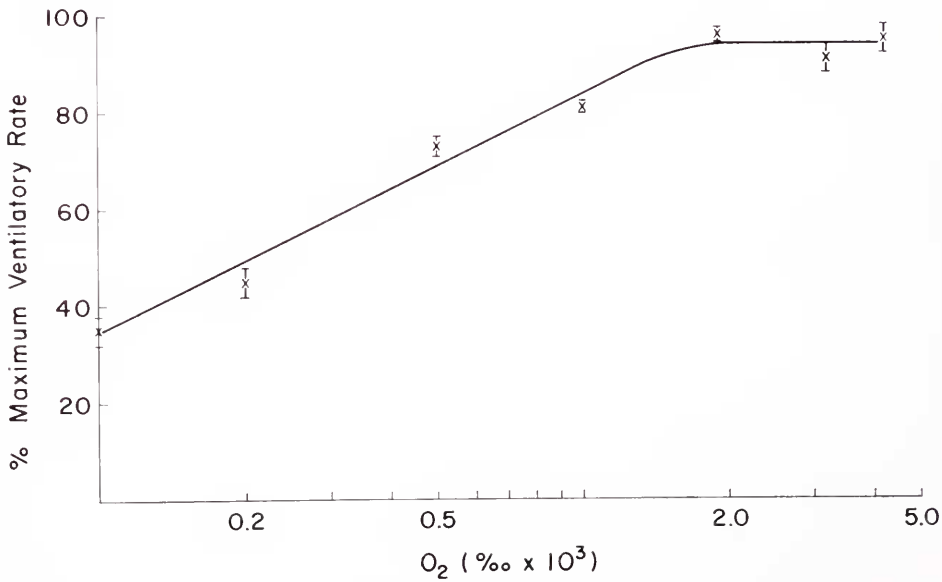

FIGURE 3. Dependence of *Limulus* ventilatory rate on the oxygen concentration of the environmental sea water. Data were obtained for four mature animals. Per cent changes in ventilatory rate were calculated for each animal. Mean values for the four animals are plotted with standard errors. Measurements were made from records obtained during four typical experimental runs in which nitrogen slowly displaced the oxygen present in air saturated sea water.

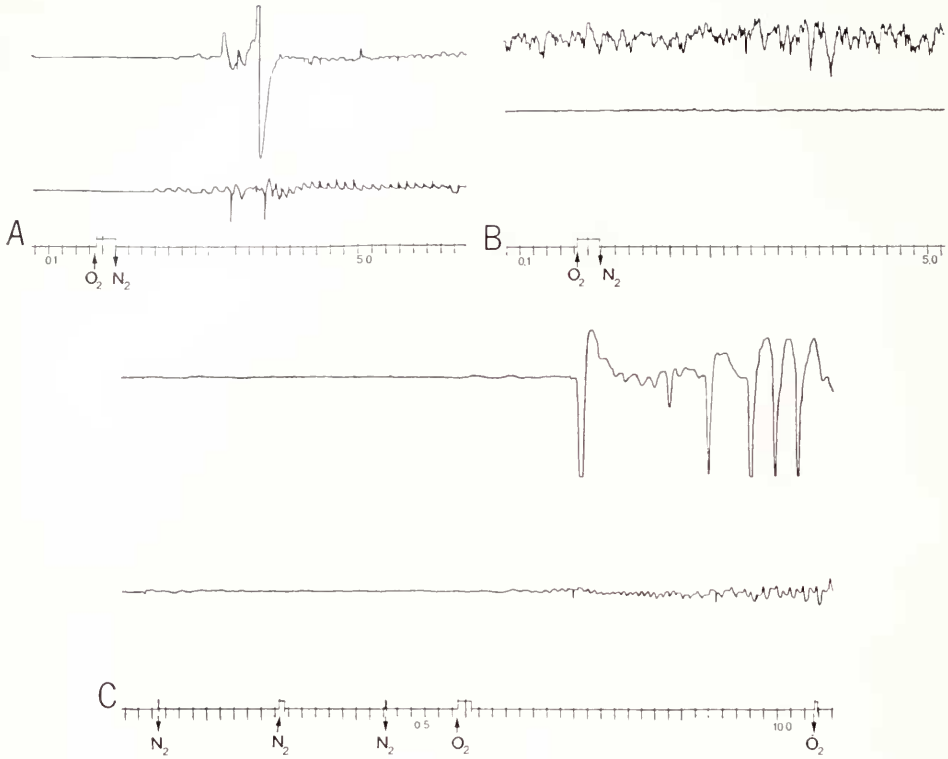

FIGURE 4. The effects of eliminating oxygen receptive structures upon *Limulus* ventilatory reflexes; (A) oxygen responsiveness of walking legs and gill appendages following removal of all book gills. Note rhythmicity of response; (B) oxygen responsiveness of animal in A (all book gills removed) following cauterization of all intercoxal sensory cuticle. Note the absence of a response to oxygen introduction; (C) oxygen responsiveness of walking legs and gill appendages following sectioning of all branchial blood sinuses. The opercular nerves and ventral nerve cord of this immature animal were previously sectioned. Note the absence of a response to turning nitrogen off and on again; upper trace: basipodite muscle responses of 5th walking leg; middle trace: adductor muscle responses in 1st gill appendage; lower trace: 5 sec time mark. All measurements of oxygen are in $\% \times 10^{-3}$. Records A and B were obtained from a mature animal with intact nerve cord. These records have been retouched for photographic reproduction.

gills. Gill appendage responses to the introduction of oxygen into the anaerobic sea water (but not to tactile stimulation) disappeared following any of these procedures: (i) section of all gill nerves which innervate the book gills; (ii) covering all book gill lamellae with wax; (iii) careful surgical removal of all book gill lamellae. The surgery usually resulted in only slight blood loss (see Methods). In contrast animals in which all branchial blood sinuses were cut open, with consequent massive blood loss, continued to initiate rhythmic respiratory movements when oxygen was introduced into the anaerobic sea water environment (Fig. 4C). Control manipulations including removal of all gill appendage endopodites and exopodites as well as covering the opisthosomal carapace with wax had no effect on oxygen responsiveness.

Prosomal responses to oxygen (but not to tactile stimulation) could be eliminated in animals with sectioned opercular nerves and ventral nerve cords by either waxing or cauterizing the membraneous cuticle between the coxa of the 5 pairs of walking legs. Removal of the coxal gnathobase spines, the flabellum or the spatulate propodite appendages of the fifth walking leg had no effect on walking leg responses to oxygen introduction. Nor was prosomal oxygen responsiveness affected by (i) cauterizing the sensory membraneous area anterior to the ventral eye; (ii) covering the ventral surface of the prosomal carapace with wax or (iii) sectioning the first dorsal nerves of the prosoma which innervate the ventral surface of the opisthosoma (Pattern and Redenbaugh, 1899).

In three animals with intact ventral nerve cords rhythmic responses of the gill appendages to the introduction of oxygen into the anaerobic chamber were reduced after waxing all book gill lamellae (Fig. 4A) and eliminated following cauterization of all prosomal intercoxal sensory cuticular areas (Fig. 4B). After removal of the wax covering the book gills rhythmic responses to oxygen introduction were restored.

Discussion

There are two morphologically different oxygen receptive systems concerned with *Limulus* ventilatory reflexes. Each provides sufficient sensory input to the central neural ventilatory pacemaking system to generate a rhythmic motor output to the gill appendages.

Oxygen sensitivity is dependent upon the integrity of the prosomal intercoxal sensory cuticle and the opisthosomal book gills. Unfortunately, consistent quantitative measurements of the behavioral effects of eliminating these structures are very difficult to obtain since: (i) the ventral nerve cord and the nerves which arise from it run within blood vessels—section of a nerve or connective produces considerable blood loss; (ii) extirpation or waxing the surfaces of the book gills drastically reduces the cuticular surface area available for respiratory gas exchange thereby leading to anoxia. However the observation of Hyde (1906) that after removal of the heart and massive blood loss—and therefore during presumed anoxia—rhythmic gill appendage movements were maintained, and the present observation (Fig. 4C) that oxygen responsiveness was not appreciably affected by cutting open all branchial blood sinuses, suggest that neither anoxia nor blood loss plays a decisive role in depressing the oxygen responsiveness of the ventilatory system.

Since neither rhythmic respiratory nor rhythmic swimming movements were ever observed after complete blockage of the prosomal and opisthosomal oxygen sensitive structures, in the intact animal afferent input from the oxygen receptive areas must be important in the maintenance of the excitation level in the central ventilatory neural network. In isolated opisthosomal nerve cord preparations Wyse (1971) has on occasion observed rhythmic output in the apparent absence of any neural input to the ventilatory rhythm generating neural network. However in the isolated single ganglion preparation, neural input is required via either the gill appendage sensory innervation (Hyde, 1906) or electrical stimulation of the nerve cord connectives (Fourtner, Drewes and Pax, 1971; Wyse, 1971).

The opisthosomal book gills are extensively innervated by the sensory gill nerve (Patten and Redenbaugh, 1899). Sensory buds and free nerve endings have been described in the book gill cuticle (Patten, 1912). However there is no

information available concerning which of these sensory elements may be the oxygen receptors, nor am I aware of any descriptions of sensory elements in the intercoxal cuticle. Information on the detailed morphology and physiology of sensory structures in the book gills as well as morphological and physiological descriptions of oxygen sensitive structures in the intercoxal sensory cuticle is presently being obtained.

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Summary

1. The rate of *Limulus* opisthosomal gill appendage respiratory movements is proportional to the logarithm of the environmental oxygen concentration. Respiratory movements cease in anaerobic sea water.

2. Oxygen introduction into anaerobic sea water elicits movement of the prosomal walking legs as well as the rhythmic gill appendage movements described by Waterman and Travis (1953).

3. The prosoma and opisthosoma each contain oxygen receptive structures. Prosomal oxygen responsiveness depends upon the sensory cuticular membranes located between the walking leg coxa. Opisthosomal oxygen sensitivity depends upon book gill integrity.

4. Sensory input from either the prosomal intercoxal cuticle and/or the opisthosomal book gills is required to generate rhythmic gill appendage movements in response to oxygen introduction into anaerobic sea water.

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